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Beech cupules share endophytic fungi with leaves and twigs

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Abstract

Endophytic mycobiota on leaves, twigs and cupules of *Fagus crenata* were investigated using a culture-dependent method over a growing season to test the hypothesis that endophytic fungi of cupule (a woody phyllome) share some components of the endophytic fungal assemblages with both leaves and twigs. A total of 14 fungal taxa were isolated, and the most frequent taxon was *Phomopsis* sp., followed by *Xylaria* sp., *Ascochyta fagi* and *Geniculosporium* sp. The compositions of fungal assemblages of leaf laminae and petioles were generally relatively dissimilar to those of current and first year twigs when compared for each sampling month, and those of cupules and cupule stalks were intermediate between those of leaves and twigs. Permutational multivariate analysis of variance confirmed that month and organ were significant factors of the variation of the composition of endophytic fungal assemblages. *Phomopsis* sp., a common twig endophyte, and *A. fagi*, a common leaf endophyte, were common in cupules and cupule stalks. These results suggested that the endophytic fungal assemblages of cupules shared component taxa with those of both leaves and twigs.

Keyword: Endophyte・*Fagus crenata*・Leaf・rRNA gene sequence analysis・Season

1. Introduction

Endophytic fungi include those that can colonize internal plant tissues at some time in their life without causing apparent harm to their host (Sieber 2007). Beech (*Fagus* spp.) is a dominant tree of cool temperate forests and has been examined for endophytic fungi, with intensive efforts devoted to Japanese beech *F. crenata* (Sahashi et al. 1999, 2000; Kaneko and Kakishima 2001; Osono 2002; Kaneko et al. 2003; Osono and Mori 2003; Kaneko and Kaneko 2004; Fukasawa et al. 2009; Hashizume et al. 2010), European beech *F. sylvatica* (Sieber and Hugentobler 1987; Danti et al. 2002) and American beech *F. grandifolia* (Chapela 1989). Most of these studies investigated endophytic fungi on leaves and twigs; but there have been no published works regarding the endophytic fungi associated with beech cupules. A cupule is a woody phyllome surrounding the seed in a fruit; thus, a cupule shares its origin with that of leaves but is chemically similar to twigs (Osono and Takeda 2001; Fukasawa et al. 2009, 2012). We hypothesized that endophytic fungal assemblages of beech cupule shared components of endophytic fungi with both leaf and twig within the shoot. The purpose of the present study was to investigate the endophytic mycobiota on leaves, twigs and cupules of *F. crenata* over a growing season to test our hypothesis.

2. Materials and methods

2.1. Study site and sample collection

Samples were collected in Ashiu Experimental Forest of Kyoto University (35°18'N and 135°43'E), Kyoto, Japan. Details of the study site were described in Osono et al. (2011). In the study site, mass flowering of *F. crenata* and mass production of cupules were observed in 2005, whereas only a few individual trees flowered in 2006. We selected a mature tree (height 16 m) that flowered in 2006, and shoots with flowers were harvested from the canopy at approx. 5–8 m height in Jun, Aug and Oct 2006. Ten shoots carrying current-year leaves, maturing cupules, a current-year twig and a one-year twig (Fig. 1) were arbitrarily selected from the canopy and harvested on each sampling date. Healthy-looking shoots without obvious faunal and/or microbial attacks were selected. The samples were placed in paper bags and taken to the laboratory.

One leaf, one cupule, two current-year twigs (1 cm in length) and two first-year twigs (1 cm in length) were taken from each shoot. The leaf was divided into lamina and petiole, and four leaf disks were punched from the lamina with a sterile cork borer (5.5 mm in diameter) from the central part of leaves, avoiding the primary vein. The cupule was cut into four equivalent pieces and one stalk. Thus, a total of 40 disks of leaf lamina, 10 petioles, 40 pieces of cupules, 10 cupule stalks, 20 current-year twigs and 20 first-year twigs were prepared on each sampling date and used for the isolation of fungi.

2.2. Fungal isolation

A surface sterilization method by Osono et al. (2008) was used for the isolation of fungi from beech organs. Fungal isolation was carried out within 24 hours of sampling. The plant organ samples were submerged in 70% ethanol (v/v) for 1 min to wet the surface, then surface-disinfected for 30 s in a solution of 15% hydrogen peroxide, and submerged again for 1 min in 70% ethanol. The samples were rinsed with sterile distilled water, transferred to sterile filter paper in Petri dishes (9 cm in diameter), and dried for 24 h to suppress vigorous bacterial growth after plating (Widden and Parkinson 1973). The leaf disks or pieces from cupules and twigs were placed in 9-cm Petri dishes containing malt extract agar (malt extract 2% w/v, agar 2%; Nacalai tesque, Kyoto, Japan), with two disks/pieces per plate. Plates were incubated at 20 °C in the dark and observed at 1, 4 and 8 weeks after surface sterilization. Identification was primarily based on micromorphological observations, with reference to Gams (2007). Some isolates were then used for molecular analysis as described below. The frequency of an individual taxon was calculated as the percentage of incidences based on the number of plant organs with the taxon relative to the total number of the organ, for each sampling date. Taxa with low frequencies were specifically discussed only if their occurrence was of special interest.

2.3. DNA analysis

Twenty-two isolates of *Phomopsis* sp., *Xylaria* sp. and *Geniculosporium* sp. were

used for DNA analysis. Thirteen isolates of *Phomopsis* sp. included seven from cupules, one from cupule stalk, two from twigs and three from lamina. Seven isolates of *Xylaria* sp. included four from cupules, two from cupule stalks and one from leaf lamina. Two isolates of *Geniculosporium* sp. included one from cupule and one from cupule stalk. Before DNA extraction, the isolates were subcultured in 2% malt extract liquid medium. The DNA was extracted from small quantities of mycelia using DNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reactions (PCR) were performed using a Quick Taq HS DyeMix (Toyobo, Osaka, Japan). Each PCR reaction contained a 50 µl mixture (21 µl distilled water, 25 µl master mix, 3 µl ca. 0.5 ng/µl template DNA and 0.5 µl each primer (final, 0.25 µM)). The primer pair ITS1f (Gardes and Bruns 1993) / LR3 (Vilgalys and Hester 1990) was used to obtain the ITS2 and the D1-D2 domain of the 28S rRNA. Each DNA fragment was amplified using a PCR thermal cycler (DNA Engine, Bio-Rad Laboratories, Hercules, USA) using the following thermal cycling schedule: the first cycle consisted of 2 min at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 50 ºC for annealing, 1 min at 68 ºC, and a final cycle of 10 min at 68 ºC. The PCR products were purified using a QiAquick PCR Purification Kit (Quiagen). The purified PCR products were sequenced by Macrogen Japan Corp. (Tokyo, Japan). The sequences determined in this study were deposited in the DNA Data Bank of Japan (DDBJ) (AB915934–AB915946, AB918138–AB918140, AB918142–AB918147). The ITS2 and 28S rRNA gene sequences were compared using MEGA5 (Tamura et al. 2011) to determine the sequences identity. All positions containing gaps and missing

data eliminated from the sequences, resulting in 884, 875 and 887 bases for *Phomopsis* sp., *Xylaria* sp. and *Geniculosporium* sp., respectively, for the comparisons. The sequences were then compared with the GenBank database using BLAST (Altschul et al. 1997).

2.4. Statistical analysis

We prepared a datasheet of endophytic fungal assemblages indicating the frequency of 14 endophytic fungal taxa on 18 samples (six organs each for three months). To compare the composition of endophytic fungal assemblages of organs within the shoot, we used nonmetric multidimensional scaling (NMDS) with the Bray-Curtis distance metric. The NMDS analysis was carried out with the *metaNDS* function with default settings of the vegan package (Oksanen et al. 2011) in the R version 3.0.2 for Mac (http://www.r-project.org). We then assessed the effect of month and organ on endophytic fungal assemblage, by analyzing the average Bray-Curtis dissimilarity matrices in permutational multivariate analysis of variance (PERMANOVA, Anderson 2001) with the *adonis* function of the R vegan package. Effects of month and organ on the frequency of four major fungal taxa (*Phomopsis* sp., *Xylaria* sp., *A. fagi* and *Geniculosporium* sp.) were analyzed with generalized linear models (GLMs) with a Poisson distribution. The GLMs were performed with the *glm* function and with the *glht* function of the R multcomp package for multiple comparisons with Tukey's test.

3. Results and discussion

A total of 14 fungal taxa were isolated from organs of *F. crenata*. The most frequent taxon was *Phomopsis* sp., followed by *Xylaria* sp., *A. fagi* and *Geniculosporium* sp. Less frequent taxa included *Epicoccum nigrum*, *Alternaria* spp., *Chaetomium* sp., *Nigrospora* sp., and six unidentified morphotaxa. *Phomopsis* sp. and *A. fagi* have been encountered in several beech forests in Japan as major endophytic fungi of twigs and leaves, respectively (Sahashi et al. 2000; Kaneko et al. 2003; Osono and Mori 2003; Hashizume et al. 2010). *Xylaria* sp. and *Geniculosporium* sp. are common Xylariaceous endophytes of leaves of multiple tree species in cool temperate forests (Osono et al. 2013; Ikeda et al. 2014) and in tropical forests (Okane et al. 2008, 2012). *Xylaria* sp. also occurs in beech twigs (Fukasawa et al. 2009, 2013). Fukasawa et al. (2012) reported frequent occurrence of *Xylaria* sp., *Phomopsis* sp. and *A. fagi* during the initial stages of cupule decomposition on the soil. In contrast, *Dasyscyphella longistipitata* and *X. carpophila*, which frequently produce fruiting bodies on dead cupules (Hosoya et al. 2010; Fukasawa et al. 2012), were not isolated from living tissues of cupules, leaves, or twigs.

The rRNA sequences of isolates of *Phomopsis* sp., *Xylaria* sp., and *Geniculosporium* sp. from different organs showed similarities between 99.7% and 100.0%, between 99.8% and 100.0% and 99.9%, respectively, indicating that the respective isolates belonged to single fungal species and that these three species had low organ specificity. Taxonomic assignment using BLAST searches

demonstrated that the base sequences of *Phomopsis* sp. had affinities to those of *P. mali* (AB665315), *P. conorum* (DQ116553), *P. fukushii* (JQ807469) and *Diaporthe eres* (JQ807441) with query coverages of 100% and max identities of 99% for all accessions. *Xylaria* sp. and *Geniculosporium* sp. were identical to ubiquitous foliar endophytes of multiple tree species in the study site (Osono et al. 2013), suggesting that these species had low levels of not only organ specificity but also host specificity.

The NMDS ordination showed differences in the endophytic fungal assemblages with respect to month and organ (Fig. 2). The compositions of fungal assemblages of leaves (leaf laminae and petioles) were generally dissimilar to those of twigs (current and first year twigs) when compared for each sampling month, and those of cupules and cupule stalks were intermediate between those of leaves and twigs. Permutational multivariate analysis of variance confirmed that month and organ were significant factors of variation of the composition of endophytic fungal assemblages (month: d.f.=2, F=6.04, P<0.001; organ: d.f.=5, F=4.20, P<0.001). These differences in endophytic fungal assemblages with respect to month and organ were chiefly attributed to the variations in the frequency of major endophytic fungal taxa as described below. Previous studies have already documented seasonal changes in endophytic fungal assemblages in tree leaves (e.g., Hata et al. 1998; Sahashi et al. 1999; Osono 2008; Osono et al. 2009).

Figure 3 shows the frequencies of four major endophytic taxa. *Phomopsis* sp. was significantly more frequent in cupules, cupule stalks and current and first

year twigs than in leaf laminae and petioles, and more frequent in Aug than in Jun or Oct. *Xylaria* sp. was more frequent in the order: cupule stalks > first year twigs > cupules > current year twigs > leaf petioles > leaf laminae, and increased from Jun to Aug and to Oct. *Ascochyta fagi* was significantly more frequent in leaf laminae and cupules than in leaf petioles and cupule stalks and in current and first year twigs, and increased from Jun to Aug and to Oct. *Geniculosporium* sp. was significantly more frequent in cupule stalks and leaf petioles than in current and first year twigs, and more frequent in Jun and Oct than in Aug.

These results supported our hypothesis and indicated that the endophytic fungal assemblages of cupules shared features with those of both leaves and twigs. One possible explanation is that cupules could serve as habitat and food suitable for the colonization of endophytic fungi associated with both leaves and twigs because cupules as woody phyllomes not only share the origin with leaves but also possess chemical similarities to twigs (Osono and Takeda 2001; Fukasawa et al. 2009, 2012). Another possibility is that cupules are located between leaves and twigs within the shoot (Fig. 1) and therefore can readily be infected by endophytic fungi of these organs, leading to the intermediate composition of endophytic fungal assemblages. In this respect, it is noteworthy that the frequency of *A. fagi* in cupules in June appeared higher than that in the other organs, including leaf laminae (Fig. 3), suggesting the advanced colonization of current year shoots by this fungal species through cupules in early months of the growing season. Such facilitated colonization may lead to higher incidence of *A. fagi* in fruiting beech shoots than in non-fruiting ones. Further studies are

needed to test whether this hypothesis is applicable to other beech trees and tree species.

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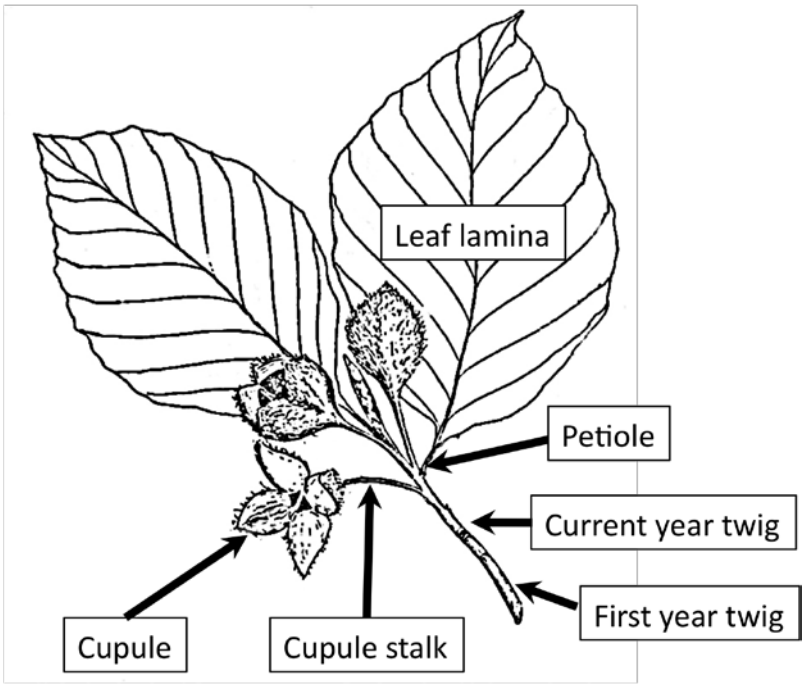
Figure legends

Fig. 1 – A fruiting shoot of *Fagus crenata*.

Fig. 2 – Endophytic fungal assemblage dissimilarity among organs examined in Jun (bold), Aug (italic), and Oct (gray), represented by nonmetric multidimensional scaling (NMDS, stress=0.155). Ll, leaf lamina; Lp, leaf petiole; Cp, cupule; Cs, cupule stalk; T0, current year twig; T1, first-year twig. The compositional dissimilarity between samples was assessed with the Bray-Curtis dissimilarity index.

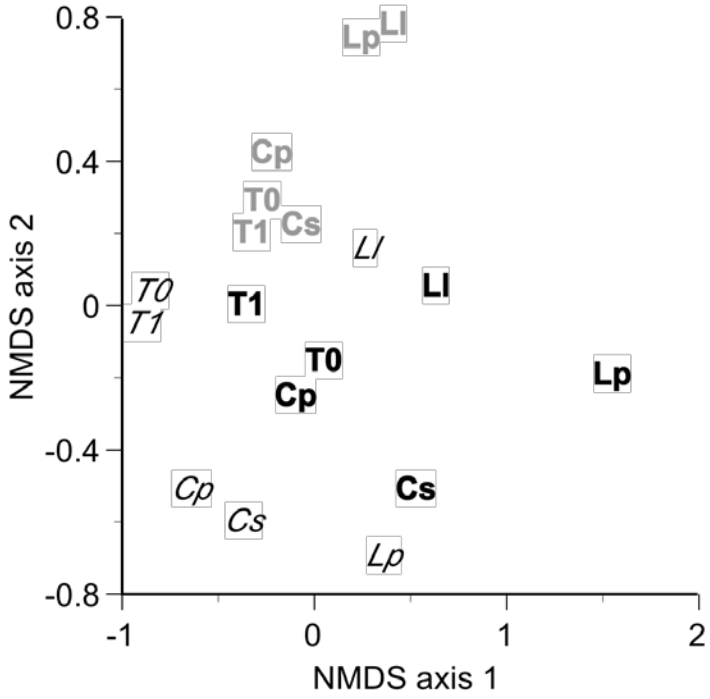
Fig. 3 – Frequency (%) of major fungal taxa on organs of fruiting shoots. Striped, Jun; shaded, Aug; blank, Oct. Ll, leaf lamina; Lp, leaf petiole; Cp, cupule; Cs, cupule stalk; T0, current year twig; T1, first-year twig. Results of generalized linear models are indicated. ***, $P<0.001$. The same letters are not significantly different between organs at 5% level with Tukey's test.

1 Tateno et al. Fig. 1



1 Tateno et al. Fig.2

2



1 Tateno et al. Fig.3